The killer toxin of the halotolerant yeast *Candida nodaensis*

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The production and secretion of killer (**K**) toxins is a widespread phenomenon in yeasts. A considerable diversity of yeast killer systems has been investigated, some of them in species considered more or less halotolerant, and which killer phenotype has been, in some cases, correlated with the degree of salt stress in the environment [1, 2, 3, 4]. In a previous survey [4], several strong **K** phenotypes were identified, some of them even displaying an enhancement of killer action in the presence of salt (Table I).

The great majority of killer toxins are considered labile proteins, unfitted for biothecnological purposes. The possibility that those yeast zymocins could be proteins less labile than the ones described so far, lead to the selection of one of those yeasts for further work. Toxins that are effective *in vivo* at high salinity are probably more stable, thus renewing the interest in these molecules for the development of yeast-based biocontrol strategies in several biotechnological applications, specially in the preservation of salted fermented foods.

Table I. Killer phenotype variation with salt concentration in the assay. Results are expressed as the percentage of killed strains, from the total assayed as sensitive, at each salt molarity. Only **K** strains which killed more than one strain were considered.

NaCl - Tolerance Class	K strains	[NaCl] in the assay (M)	S strains (%) 0	0.5	1	1.5	2	2.5	3	3.5
		Number of strains tested for S phenotype (100%)	57	56	47	45	20	20	7	5
1 M	*P. jadinii*		38.6%	12.5%	0	n.a.	n.a.	n.a.	n.a.	n.a.
	Z. florentinus		5.3%	7.1%	6.4%	n.a.	n.a.	n.a.	n.a.	n.a.
2 M	*K. lactis* CBS 2359		26.3%	32.1%	4.3%	0	0	n.a.	n.a.	n.a.
	P. anomala IGC 4121		35.1%	48.2%	46.8%	44.4%	50.0%	n.a.	n.a.	n.a.
	S. cerevisiae IGC 4620		8.8%	8.9%	10.6%	4.4%	0	n.a.	n.a.	n.a.
3 M	*C. famata*		0	1.8%	21.3%	28.9%	30.0%	15.0%	0	n.a.
	D. hansenii		14.0%	23.2%	34.0%	35.6%	40.0%	35.0%	0	n.a.
	P. farinosa		10.5%	21.4%	21.3%	13.3%	5.0%	0	0	n.a.
	P. membranaefaciens IGC 3796		8.8%	3.6%	0	0	0	n.a.	n.a.	n.a.
4 M	*C. nodaensis*		12.3%	21.2%	34.0%	35.6%	40.0%	25.0%	0	0
	C. versatilis		3.5%	3.6%	4.3%	6.7%	15.0%	5.0%	0	0
	P. sorbitophila		5.3%	17.9%	17.0%	11.1%	0	0	0	0

One of the strongest **K** strains identified above (Table I), the halotolerant yeast *Candida nodaensis* IGC 3198, was chosen for further study and toxin purification.

In order to identify the genetic determinant of this **K** toxin, some curing experiments (Table II) and nucleic acid extractions (Fig. 1) were performed. The absence of linear DNA plasmids and the impossibilitie of curing with some known curing agents suggested that this zymocin is encoded by a nuclear gene.

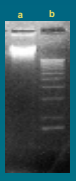

Fig. 1. Nucleic acid extractions from *C. nodaensis* (a). (b) Molecular weight marker X (Boehringer)

Table II. Curing experiments performed in *C. nodaensis*.

Cicloheximide %	% curing	Temperature (ºC)	% curing	Etidium bromide % curing
0,001	**0**	35	**0**	**0**
0,01	*	37	**0**	
		40	*	

Thus the following strategy was the isolation and purification of this yeast **K** factor, now in progress in our laboratories.

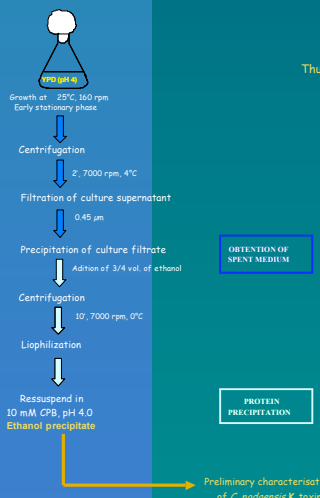

Fig. 2. Experimental protocol developped to obtain *C. nodaensis* **K** toxin

Different media and culture conditions were tested to optimise the prodution of *Candida nodaensis* **K** toxin. Culture supernatant was concentrated by ethanol precipitation (Fig. 2) and used to preliminary characterize this **K** factor (Table III and Fig. 3, 4, 5).
K activity was tested against the sensitive strain *Pichia guilliermondii*, in methylene blue agar plates (YMA-MB) with or without NaCl.

Table III. K activity of an ethanol precipitate after different enzimatic treatments (20h, 20ºC). Control experiments were done with identical samples without the respective enzymes (100% K activity).

Enzimes	Residual K activity: Active	Inactive
Endonucleases		
DNase	100%	100%
RNase	100%	100%
Proteases (2mg/ml)		
Proteinase K	**86%**	100%
Tripsin	100%	100%
Pronase	100%	100%
Papain	100%	100%
Pepsin	**76%**	100%

Previous results showed that this toxin was stable after 15' incubation at temperatures varying from -80ºC to 45ºC. Three temperature values were selected to further characterise *C. nodaensis* K factor (Fig. 3, 4, 5).

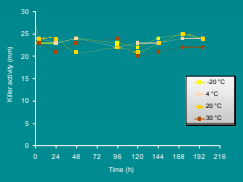

Fig. 3. K activity after 178h incubation at -20ºC, 4ºC, 20ºC and 30ºC.

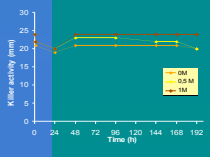

Fig. 5. Effect of the presence of NaCl (0, 0.5 and 1M) on K toxin stability.

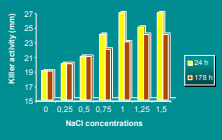

Fig. 4. Effect of the presence of NaCl in the assay medium.

The majority of the **K** toxins are considered labile proteins, unfitted for biotechnological purposes. Preliminary experiments performed to characterise *C. nodaensis* K factor showed that, besides keeping its biological activity at higher NaCl concentrations, is stable after incubation in a relatively broad range of temperature (up to 45ºC) and pH values (2.5 to 7.5). Along with the purification of this protein, the study of its mode of action, will certainly contribute to evaluate its biotechnological potentialities in the preservation of high-salt food products.

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(2) **Llorente, P., Marquina, D., Santos, A., Peinado, J. M. and Spencer-Martins, I.** (1997). Effect of salt on the killer phenotype of yeasts from olive brines. *Appl. Environm. Microbiol.*, 63: 1165-1167.
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(4) **Aguiar, C. and Lucas, C.** (2000) Killer/sensitivity phenotypes and halotolerance. *Food Technol. and Biotechnol.*, 38 (1): 39-46.